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DISINFECTION OF VEGETATIVE CELLS
OF *BACILLUS ANTHRACIS*

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Disinfection kinetics of vegetative cells of *Bacillus anthracis* in water with free available chlorine ([FAC] 2 mg/L) and monochloramine ([MC] 2 mg/L) were established in this study. FAC disinfection was performed in chlorine demand-free phosphate buffer at pH 7 and 8 at two temperatures (5 and 25 °C). MC disinfection was performed in normal phosphate buffer at pH 8 at both temperatures. FAC was more effective than MC in causing cell death, which was more rapid at 25 °C than at 5 °C at both pH 7 and 8. For MC disinfection, the cell inactivation rate was more rapid at 2 °C. The disinfection kinetics were rapid within the first 5 min, followed by a slow cell inactivation. The results were complicated by the varying number of spores present in the test inoculums. Although protocols were developed and implemented to minimize the spore number, varying numbers of spores were observed in the different runs. The presence of spores also resulted in high variability, especially at sublethal exposure levels. Our efforts were focused on developing a new culture recipe (RVLS) to ensure a minimal number of spores and to confirm the absence of spores in the sporulation-minus strain (spo⁻) of *B. anthracis*.

Bacillus anthracis cells	Drinking water	Chlorine demand-free (CDF)
Free available chlorine (FAC)	Monochloramine (MC)	Sporulation-minus strain (spo⁻)

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PREFACE

The work described in this report was authorized under U.S. Environmental Protection Agency, National Homeland Security Research Center, Water Infrastructure Protection Division. This work was started in November 2012 and completed in October 2014.

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DISINFECTION OF VEGETATIVE CELLS OF *BACILLUS ANTHRACIS*

1.   INTRODUCTION

Disinfection of *Bacillus anthrac*is spores in drinking water is well documented in peer-reviewed literature (Adcock et al., 2004; Raber and Burklund, 2010; Rogers et al., 2006; Rose et al., 2005; Rose et al., 2007; Sivaganesan et al., 2006; and Szabo et al., 2012). Vegetative bacterial cells are generally sensitive to free chlorine, and chlorine demand is dependent on initial cell number, temperature, and organic contaminants (Helbling and Vanbriesen, 2007 and Johnson et al., 1997). *B. anthracis* vegetative cells are assumed to be highly susceptible to inactivation with common disinfectants such as free chlorine. However, disinfection studies supporting this assumption and data on chlorine demand for inactivation of these cells are lacking. A recent study, jointly performed by U.S. Army Edgewood Chemical Biological Center (ECBC) and U.S. Environmental Protection Agency (EPA) members, has established the persistence of *B. anthracis* cells for more than a month in dechlorinated water. In addition, the joint ECBC and EPA team has shown that temperature and organic contaminants affect the decay of such cells. Our goal was to perform inactivation studies using vegetative *B. anthracis* cells, which will help in determining the inactivation of biowarfare agents in a contaminated water distribution system and the fate of vegetative cells resulting from augmented germination.

In this study, data were generated on the inactivation of vegetative *B. anthracis* cells in water using common disinfectants (i.e., free available chlorine [FAC] and monochloramine [MC]). A surrogate of the pathogenic Ames strain of *B. anthracis*, *B. anthracis* (ΔSterne) was used in this study. The impact of changing water conditions (i.e., pH and temperature) was assessed. The effects of two holding temperatures (5 and 25 ºC) at pH 7 and 8 were evaluated on the inactivation of cells. The efficacies of FAC at 1–2 mg/L and MC at 2 mg/L for disinfecting water were evaluated.

2.   MATERIALS AND METHODS

2.1   Strain Procurement and Selection

An avirulent plasmid-free strain of *B. anthracis* ([ΔSterne] BaDS) was obtained from the ECBC BioDefense Branch. Colony morphology, biochemical and physiological tests, and genetic testing were performed to confirm the validity of the culture. BaDS is an avirulent strain in which the pXO1 and pXO2 plasmids are missing.

2.2   Culture Maintenance and Inoculum Preparation

The BaDS strain was grown at 37 ± 2 ºC on tryptic soy agar (TSA) plates. For the primary culture, a single colony from a freshly inoculated TSA plate was inoculated in sterile tryptic soy broth (TSB) media and incubated at 37 ± 2 ºC for 24 h. After 24 h, a 50 mL secondary culture was initiated by inoculating fresh sterile TSB media. An aliquot of 100 μL of the overnight culture was used to inoculate 10 mL of TSB. The culture was incubated at 37 ± 2 ºC for 4 h.

2.3 Cell Preparation

For the first wash (wash 1), the cell suspensions of BaDS were centrifuged for 10 min at 3000× g, the supernatant was discarded, and the pellet was resuspended in 25 mL of sterile chlorine demand-free (CDF) buffer at pH 7 or 8. The cells were again centrifuged as before, and the pellet was resuspended in 25 mL of sterile CDF buffer two more times (washes 2 and 3). After a fourth spin, the pellet was resuspended in 20 mL of CDF buffer, placed on ice, and used as test inoculums within 60 min.

2.4 Disinfectant Preparation

FAC solution was prepared following some modified methods provided by EPA members. All the glassware, stirrers, and solutions were made to be CDF. A 0.05 M potassium phosphate buffer was prepared at pH 7. Sodium hypochlorite was first diluted by 1:200 and used the same day. Free chlorine solution (2 mg/L) was prepared by appropriate dilutions of 1:200 diluted sodium hypochlorite solution with phosphate buffer at pH 7.

MC was prepared in accordance with the modified EPA method. Phosphate buffer at pH 8.3 was prepared and used for all the dilutions. First, a solution of 1000 mg chlorine solution was prepared in two steps. Sodium hypochlorite solution was diluted 1:5, and then 1:10. Secondly, 1000 mg/L of ammonia nitrogen was prepared by dissolving 0.472 g of ammonium sulfate powder in 100 mL of water at pH 8.3. Finally, an aliquot of 50 µL of nitrogen and an aliquot of 200 µL of chlorine solution were added to 100 mL of phosphate buffer at pH 8.3. This solution was measured to yield a concentration of 2 mg/L of MC using the free and total chlorine concentrations.

2.5 Spiking Holding Solutions

For FAC, 198 mL of CDF phosphate buffer was equilibrated to test the temperature in a borosilicate glass beaker and then used for the experiments. For MC, 198 mL of "normal" phosphate buffer was used to conduct the disinfection runs because CDF buffer does not sustain MC concentration. Stir plates were used for constant mixing of solutions. Before the FAC tests, measured volumes of pH 7 or 8 CDF buffers were added to two large beakers, respectively. Reagent-grade FAC solution was then added to the beakers to obtain the desired initial chlorine concentration. For the MC tests, pH 8.3 phosphate buffer and MC were similarly added to a large beaker. Free and total chlorine concentrations were determined using a Hach Company (Loveland, CO) pocket colorimeter II analysis system for chlorine (Hach catalog number 59570-88). This was done to perform the diethyl-p-phenylenediamine (DPD) colorimetric method, which is equivalent to Standard Method 4500-CI G for drinking water. Aliquots of the chlorinated buffer were transferred to three sterile CDF beakers. One beaker was used as a control to monitor chlorine levels throughout the test sampling, and the other two were used for replicate test samples. In addition, two control beakers were used for CDF buffer for FAC and MC phosphate buffer, respectively. Throughout the testing, a stir bar was used to determine the initial titer of the BaDS cells and the titer in a nonchlorinated environment.

The labeled test beakers and the titer control beaker were spiked with BaDS cells (2 mL washed cells) to yield an initial titer of 6–7.5 log10 colony-forming units (cfu)/mL. A

National Institute of Standards and Technology traceable timer was used to monitor incubation times, as soon as the bacterial suspensions were added. The suspensions were stirred constantly throughout the sampling times. Aliquots of 10 mL were removed from the test and control beakers, and the disinfectant was neutralized by transferring the aliquots to tubes containing 0.10 mL of 10% (wt/vol) sterile sodium thiosulfate at each predetermined exposure time (±5 s). Separate aliquots were removed from a control beaker at the beginning and end of an experiment to measure the chlorine concentration of the solution. Tests were conducted in two different incubators, which were set at 5 and 25 ºC, respectively. The reaction vessels for each test consisted of four sterile CDF borosilicate glass beakers, one each for FAC and phosphate buffer for MC. Each reaction vessel contained a CDF stir bar. Stir plates were used for constant mixing of the solutions. Prior to the test, a measured volume of pH 7 CDF buffer was added to a large beaker, and 8.0 CDF buffer was added to another large beaker. Reagent-grade sodium hypochlorite solution or MC was added to the beakers to obtain the desired initial chlorine concentration. Free and total chlorine concentrations were determined using the Hach pocket colorimeter II analysis system for chlorine to perform a DPD colorimetric method, which corresponds to Standard Method 4500-CI G for drinking water.

Initial densities and the numbers of surviving cells were determined using the spread-plate method, which is described in Standard Method 9215C. One tenth of 1 mL aliquots of appropriate dilutions were dispensed and spread on TSA plates. In addition, a 10 mL aliquot of appropriate dilution was filtered through a 0.2 μm filter and placed on a TSA plate. The plates (for each dilution were incubated for 20 + 4 h at 36 ± 1 °C, and the colonies were counted with the aid of a Qcount colony counter (Advanced Instruments, Inc., Norwood, MA) . For samples with very low viable cell counts, a 10 mL aliquot was filtered and the colonies that appeared on the filter were overlaid on a TSA plate and used to estimate the residual viable cell number. A cellulose nitrate Nalgene (Thermo Scientific, Rochester, NY) analytical filter unit (47 mm with 0.2 μm pore size) was used for filtering the test water. This ensured a low limit of detection (LOD) through the study. For the entire study set, the LOD was >1–5 viable cells.

We took a pH reading of the CDF and phosphate buffers before beginning the control run, and then, we took another pH reading of the solutions in the test beakers at the conclusion of the run. Total and free chlorine measurements were taken from the chlorine control beaker at time zero and at the last exposure time.

3.   RESULTS

3.1   Test Inoculum Preparation: Vegetative Cell Preparation with Low Spore Numbers

Because this study was focused on the disinfection of vegetative cells, the first challenge was to use culture conditions, which could be used for high vegetative cell growth (>8 log/mL) while maintaining spore number as close to zero as possible (<2 log). The two factors found to be most critical in controlling sporulation onset were C/N abundance/media type and growth temperature.

The base media for culturing BaDS cells was TSB. Culturing media for optimizing high vegetative cell numbers with minimal spore population included 2× TSB,

3× TSB, 2× TSB (supplemented with carbon or nitrogen), J media, and a new recipe (ECBC media for minimizing sporulation) conceived and developed by Vipin Rastogi and Lisa Smith (RVLS). Ideally, the goal was to obtain no spores or a minimal number of spores in the 4 h cultured broth, while keeping a high cell number. A preparation with high ratio (>5–6) was considered suitable for use in the proposed set of experiments. The relationship could be expressed as a ratio of log cells versus log spores for a given preparation. Figure 1 summarizes the results in terms of the ratio, and as can be observed, high ratios were obtained by growing the broth culture at 30 ºC or using other media. Finally, sporulation-minus (*spo*⁻) derivative of BaDS was acquired from Steve Leppla (National Institutes of Health [NIH], Bethesda, MD) and tested for the presence of spores (data not shown). Absence of spores in the *spo*⁻ strain was confirmed. This was the best choice of strain to use for future experiments with vegetative cell disinfection.
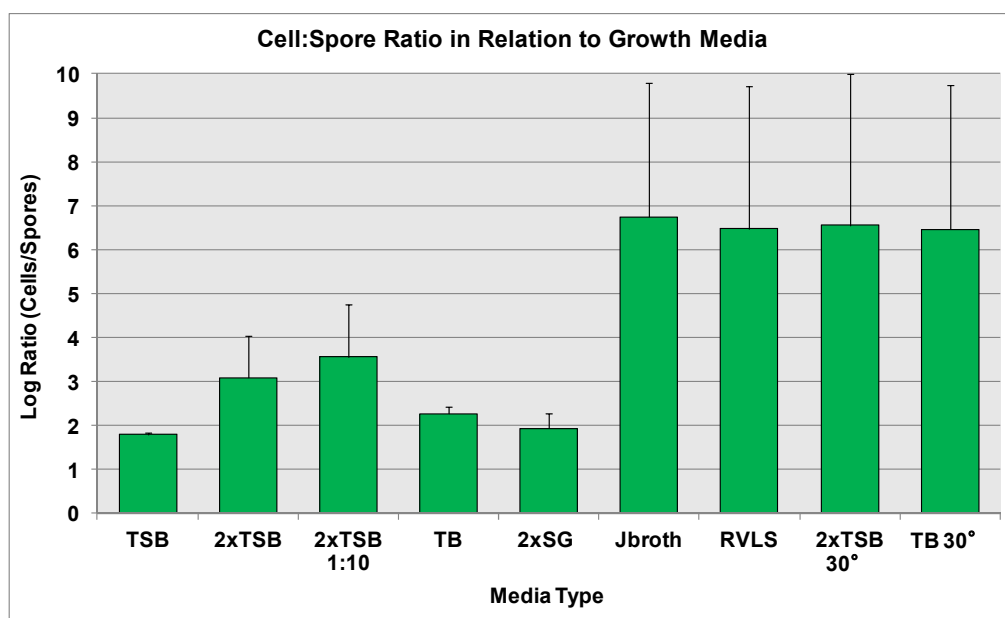

Figure 1. Spore ratio in cell preparations with different media.

3.2   Results of Disinfection Study

3.2.1   FAC Disinfection

During a 7 month period, 5 experiments at pH 7 and 4 experiments at pH 8 were completed. At 5 ºC, the change in FAC (ΔFAC) at pH 7 varied between 0.5 and 1 mg/L, and at pH 8, it varied between 0.25 and 0.6 mg/L. At 25 ºC, the change in FAC at pH 7 varied between 0.5 and 1.5, and at pH 8, it varied between 0.4 and 0.85. A wide range in FAC changes could partly be caused by varying spore numbers or residual media-component contaminants in each run.

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3.2.2 Correlation between ΔFAC and Δcfu during the Run

The data did not appear to support the idea of a correlation between ΔFAC over the run course and the log reduction or change in viable cell numbers at the end of a 30 min test run (Figure 2).
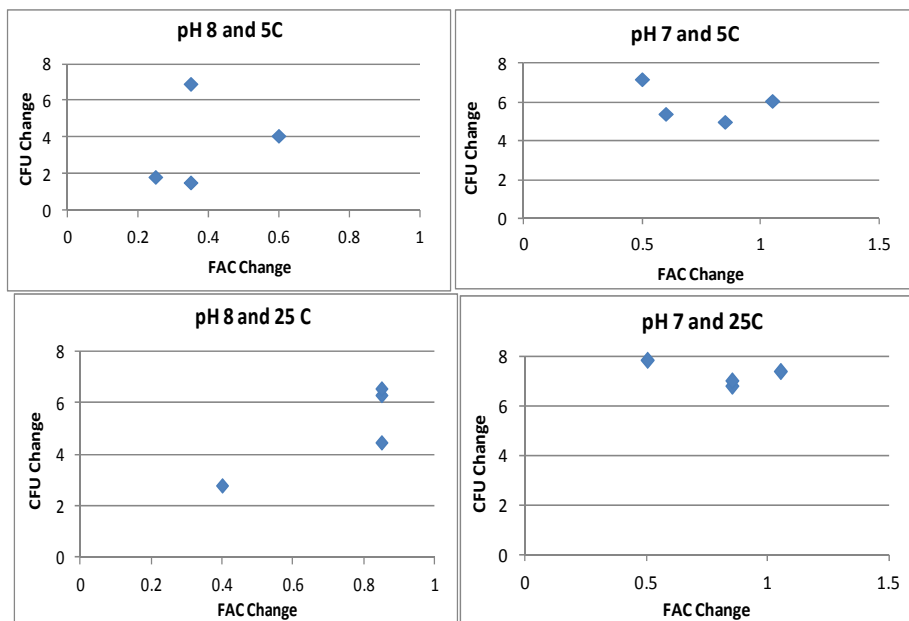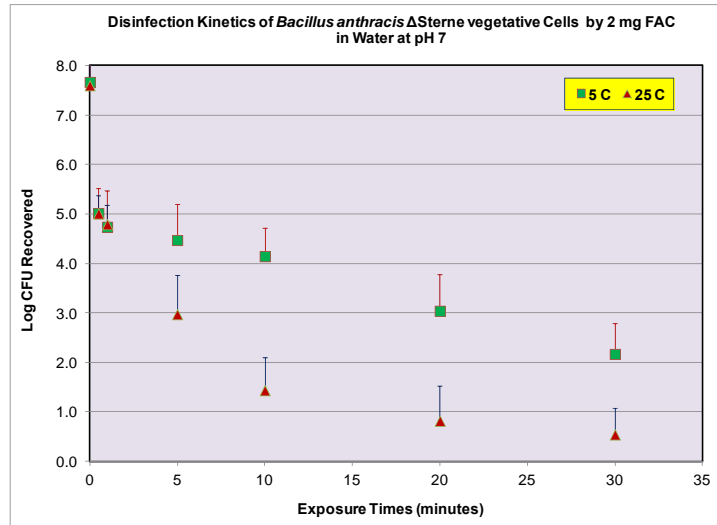

Figure 2. Correlation between ΔFAC and Δcells.

3.2.3 Disinfection Kinetics at pH 7

Of the four experiments, one appeared to be an outlier (i.e., run 2, in which ~6 log kill occurred within the first 30 s for no apparent reason [initial FAC or initial cfu number, temperature, or FAC consumption]). Two plots are shown in Figure 2: one with three experiments (excluding run 2) and the second with all four runs included.

The kinetics plots are shown in Figure 3. As expected, higher standard deviation (SD) was observed when all four experiments were included. Two clear trends are described in the following sections.

A

Disinfection Kinetics of *Bacillus anthracis* ΔSterne vegetative Cells by 2 mg FAC in Water at pH 7

B

Disinfection Kinetics of *Bacillus anthracis* ΔSterne vegetative Cells by 2 mg FAC in Water at pH 7
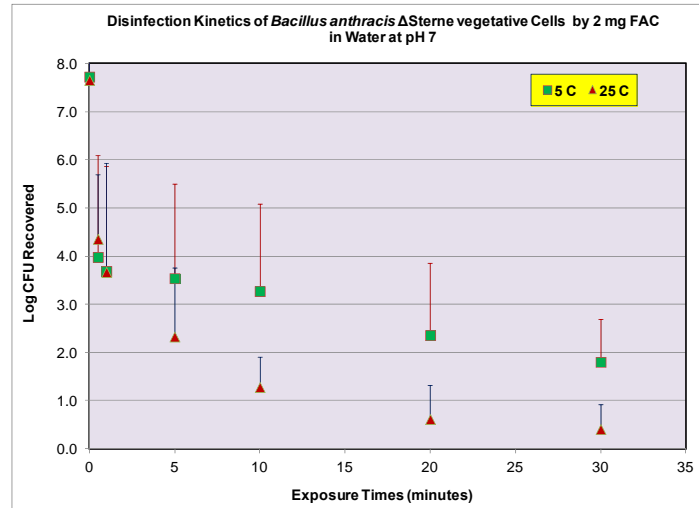
Figure 3. Disinfection kinetics at pH 7: (A) data exclude
second experiment and (B) data include all four experiments.

Within 30–60 s exposure of FAC in both plots shown in Figure 3, a 2–3 log reduction is evident, and the kill rate significantly slowed with longer exposure periods. The persistent cell population (0.5–2 logs) appears to represent the spores added at the start of the disinfection run. First, the low level of FAC (1 or 2 mg/L) had very little to no effect on the viability of the persistent spore population. Second, the loss of cell viability in response to FAC appeared to be rapid at 25 ºC, compared with that at 5 ºC.

3.2.4   Disinfection Kinetics at pH 8

Figure 4 summarizes the disinfection kinetics of vegetative cells at pH 8. Compared with pH 7, where the kill kinetics were a lot faster at 25 °C than at 5 °C, the kill

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kinetics at pH 8 were similar at both test temperatures. The residual lingering population largely consisted of spores that were not inactivated under the test conditions. The SD was significantly high (1–3 log). It appears that a persistent population may have largely represented the spore fraction (reducing the spore number added to the test beaker would be highly desirable).
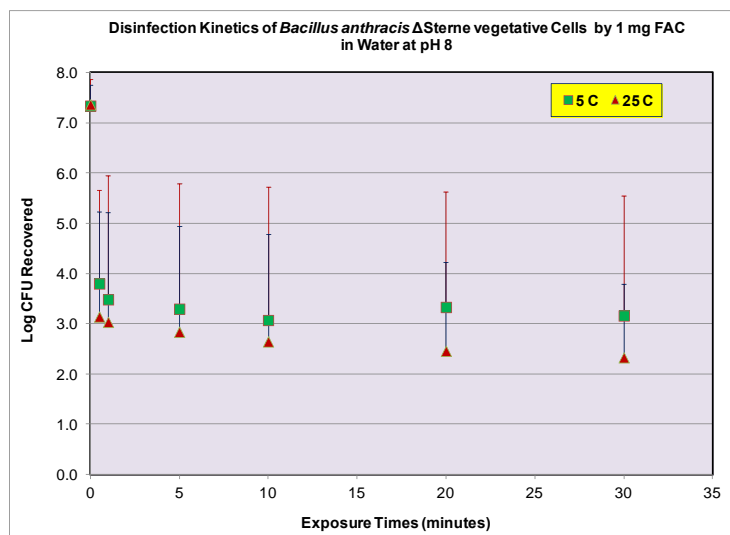

Figure 4. Disinfection kinetics at pH 8.

3.3   MC Disinfection

Although a number of experiments were performed with MC at pH 8.3, only four of them are included in this report. The other experiments were excluded because of an excessive number of spores (>3 log) or a low number of vegetative cells (<6 log/mL) in the findings. The data showed no correlation between change in MC and cell numbers at 5 ºC. At 25 ºC, the change in MC concentration was almost zero or within the noise level. Two factors could have contributed to a noticeable change in MC: (a) variable spore numbers and (b) organic media contaminants included with the washed cells. In general, an inverse relationship was observed between the ΔMC and Δcells (Table 1 and Figure 5).

Table 1. MC Concentration at Start and End of Run and Change in Cell

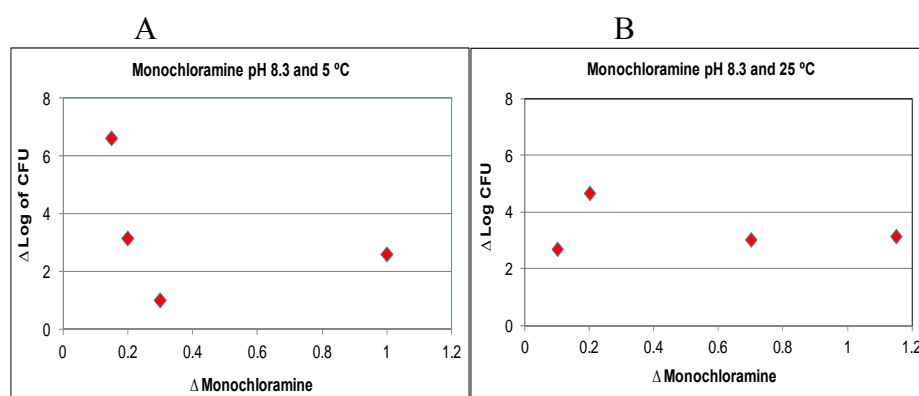|		5 ºC		25 ºC		5 ºC	25 ºC	5 ºC	25 ºC
Dates	pH	MC (Start)	MC (End)	MC (Start)	MC (End)	Δ MC	Δ MC	Δ Log cfu (0–End)	Δ Log cfu (0–End)
4/10/14	8.3	2.00	1.60	1.20	0.65	0.4	0.55	1.035	3.17
7/15/14	8.3	1.55	1.40	1.35	1.50	0.15	−0.15	3.18	2.72
7/17/14	8.3	1.60	1.10	2.05	1.10	0.5	0.95	2.63	3.05
7/22/14	8.3	1.65	1.55	1.45	1.50	0.1	−0.05	6.65	4.69

A   B

Figure 5. Correlation between ΔMC and Δcells.

Figure 6 summarizes the disinfection kinetics of vegetative cells in the presence of 2 mg/mL of MC at pH 8.3. The kill kinetics appeared to be slightly more accelerated at 25 °C than at 5 °C. Overall, compared with FAC disinfection, the kill kinetics in the presence of MC appeared to be slow, with a high residual amount of disinfectant remaining at the end of the run. The presence of a residual amount of FAC could also have contributed to cell inactivation. The solid line shows average logs of spore numbers in this set of experiments. The SD values were all <1.5 log, which does not appear to be high, considering the nature of experiments under sublethal kill conditions.
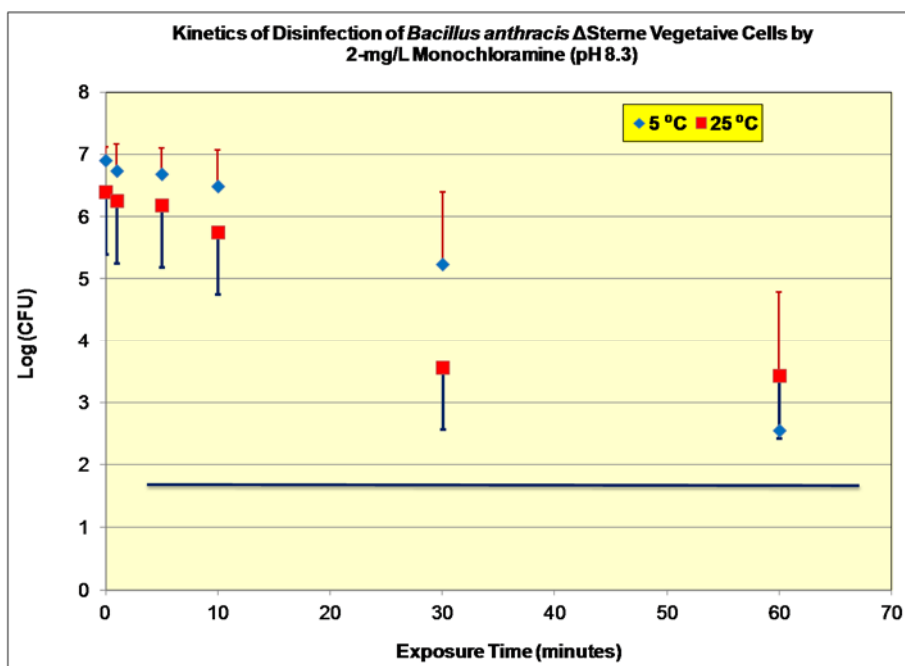
Figure 6. MC disinfection kinetics at pH 8.3.

3.4   FAC Disinfection: Conclusions and Future Recommendations

In general, FAC disinfection was more rapid at 25 ºC and pH 8. In fact, the kinetics of cell disinfection at pH 8 at the two temperatures were comparable. If all of the analyzed data are considered, high variability and high SD values are evident.

Even though a great deal of effort was taken to control preparation of cells, variability leading to high SD in CDF water, disinfectant, and test runs was evident in disinfection kinetics. One uncontrolled variable was the number of spores present in the cell preparation in the different runs. Because the media used for culturing the cells was undefined (yeast extract, tryptone, or peptone), the precise components of each batch of media were unknown. Furthermore, even for TSB and the same growth protocol, spore number varied from low (<1 log) to high (>3 log). In future experiments, using a spo⁻ strain with zero spores in cell preparation is expected to reduce the variability arising from fluctuating chlorine demand resulting from the difference in spore numbers. To this end, a new growth media recipe was conceived and tested for the parent strain. Figure 7 summarizes the spore number calculated for each run at the start and end of the disinfection runs. The spore number remained the same (<0.3 log) within a run at the start and end of a run, but varied vastly (1–2 log) from run to run. Even though a slight difference (0.2–0.5 log) was observed, the spore numbers were comparable.
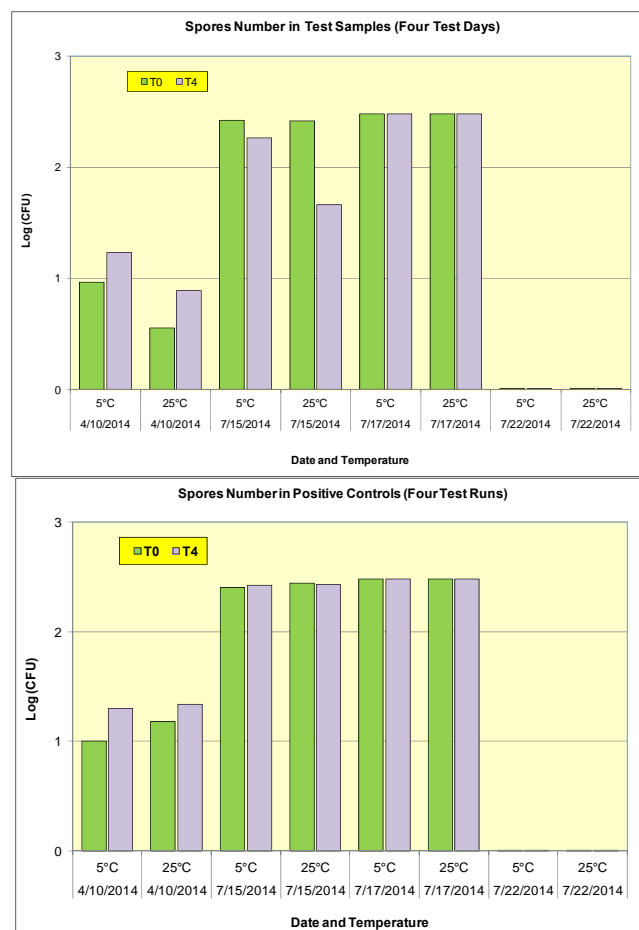
Figure 7. Spore abundance in test and control samples.

4.   DISCUSSION AND CONCLUSIONS

The key objectives of this study were twofold: (a) to determine the disinfection kill kinetics of FAC against vegetative cells of *B. anthracis* and (b) to determine the disinfection kill kinetics of MC against vegetative cells of *B. anthracis*. At a sublethal exposure of the disinfectant, variability in the test results was expected. Observed significant variability (>2–3 log) was contributed by the following factors:

- the variable number of spores in the different vegetative cell preparation batches;
- the residual amount of organic media carried over with the washed cells; and
- for MC, the presence of a residual amount of FAC.

In general, cells appeared to be more sensitive to FAC (2 mg/mL) than to MC (2 mg/mL). The rate of kill for FC was rapid (within 30 min) as opposed to that of MC (within 60 min). Disinfection by FAC was more rapid at 25 ºC than with that at 5 ºC. Relatively, a higher residual amount of MC than FAC was recorded at the end of the test run. Because MC

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disinfection tests were only conducted at pH 8, it appears that MC was even less efficacious at neutral pH. These general trends are clearly evident, even against high variability.

Table 2. Cell and Spore Numbers in Different Growth Trials

Media Type	Average Cell No.	Average Spore No.
3× TSB (200 µL)	7.97	2.67
3× TSB (500 µL)	8.36	3.08
2× TSB	7.57	4.05
3× TSB 1:10	5.09	0
2× TSB plus1% ammonium sulfate	7.68	2.65
1× TSB plus1% ammonium sulfate	7.49	4.48
TSB nitrogen	7.29	3.48
TSB nitrogen and carbon	7.9	3.48
2× TSB nitrogen	6.3	3.48
2× TSB nitrogen and carbon	7.51	3.08
TSB original	8.49	4.72
1:10 TSB	7.78	4.63
1:100 TSB	6.87	4.41
1:10 2× TSB	7.18	1.65
1:100 2× TSB	5.62	0.97
2× SG	8.06	4.24
2× SG 1:10	6.93	3.64
TB	8.73	3.87
TB 1:10	8.02	3.32
J media 20 mg glucose	8.75	3.79
J media 50 mg glucose	8.61	1.16
J media 100 mg glucose	8.60	1.50
RVLS	8.35	1.01
2× TSB 30 ℃ 4 h	6.82	0
2× TSB 30 ℃ 6 h	8.39	1.72
2× TB 30 ℃ 4 h	7.65	0
2× TB 30 ℃ 6 h	8.41	1.72

SG, sporulation growth.
TB, terrific broth.

One observation that confounded us was the variation in spore numbers, even when equal amounts of growth media and protocol were used. Sporulation is a complex process, and it is triggered by stresses related to nutrient deprivation, nitrogen content, and cell division, as determined by the number of cells used to initiate the 4 h culture. After the disinfection runs were completed, our efforts were focused on controlling the extent of sporulation. Two general approaches were followed to address the issue of spores in cell preparation. First, different media (Table 1) were included to see how they affected the number of spores (Figure 7). Table 2

summarizes cell number relative to spore number, as affected by media and inoculation condition. A *spo⁻* strain was procured from NIH, and the phenotype of this strain was confirmed to contain no spore formation, irrespective of the growth period. In addition, in this study, a new culture media was conceived and tested to yield a very low number of spores, even with the parent strain.

To further substantiate the efficacy of FAC and MC disinfection and generate kinetic runs with <1 log SD variability, we recommend that this study be repeated with the use of the *spo⁻* strain of *B. anthracis* or the new growth media, RVLS. This media appears to support minimal sporulation, thereby controlling the number of spores in cell-preparation batches in different runs.

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ACRONYMS AND ABBREVIATIONS

BaDS	*Bacillus anthracis* ΔSterne
CDF	chlorine demand-free
Cfu	colony-forming unit
DPD	diethyl-*p*-phenylenediamine
ECBC	U.S. Army Edgewood Chemical Biological Center
EPA	U.S. Environmental Protection Agency
FAC	free available chlorine
LOD	limit of detection
MC	monochloramine
NIH	National Institutes of Health
RVLS	ECBC media for minimizing sporulation
SD	standard deviation
SG	sporulation growth
TB	terrific broth
TSA	tryptic soy agar
TSB	tryptic soy broth

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